IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Application of Kathleen C.M. Campbell — Art Unit 1614
Serial No. 10/694,432
Filed October 27, 2003
Confirmation No. 8934
For THERAPEUTIC USE OF D-METHIONINE TO REDUCE THE TOXICITY OF RADIATION
Examiner Rebecca Cook

DECLARATION OF KATHLEEN C. M. CAMPBELL UNDER 37 CFR 1.132

I, Kathleen C.M. Campbell, hereby declare and state as follows:

1. I reside at 11941 Clearspring Drive, Glenarm, Illinois 62536.

2. I received a Doctor of Philosophy in Audiology/Hearing Science from the University of Iowa in 1989.

3. I am currently a Professor and the Director of Audiology Research in the Division of Otolaryngology, Department of Surgery at the Southern Illinois University School of Medicine in Springfield, Illinois.

4. I am the named inventor of the subject application, which claims methods for treating or preventing ototoxicity, neurotoxicity, alopecia, gastrointestinal disorder, or reduced survival in a patient exposed to radiation for a time and at an intensity sufficient to result in ototoxicity, neurotoxicity, alopecia, gastrointestinal disorder, or reduced survival.

5. Under my direction and control D-methionine was evaluated as a protective agent for various side effects of radiation. Generally, we used 5 control animals that were not exposed to radiation, 5 animals that were injected with saline 30 minutes prior to radiation exposure, and 5 animals that were

injection with 300 mg/kg of D-Methionine 30 minutes prior to radiation exposure. Radiation exposure was delivered in a single dose of 70 Gy (7 krad) port-limited radiation to the cranium across the cochleae. Auditory brainstem response (ABR) threshold levels were tested pre- and post-radiation. Twenty-four hours after radiation exposure cochlear levels of antioxidant enzymes were analyzed. D-methionine significantly protected glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), and catalase (CAT) activity levels.

6. The animals used for this study are described below. Complete data sets were obtained for three groups of five male Wistar rats (250-350 g) each. All animals were anesthetized with 1ml/mg i.m. of Rompun cocktail (a solution containing 86.21 mg/ml ketamine and 2.76 mg/ml xylazine) prior to radiation and testing. Anesthesia was supplemented as needed with half doses throughout testing. The three groups included: (1) a control group which received no radiation, (2) a group that received a single injection of saline 30 minutes prior to radiation, and (3) a group that received an i.p. injection of 300 mg/kg of D-Methionine 30 minutes prior to radiation. Radiation consisted of 70 Gy of ionizing radiation, an amount equal to a common cumulative clinical dose for head and neck cancer. All animals were sacrificed 26 to 27 hours after radiation, while still anesthetized from final auditory brainstem responses (ABRs). Sacrifice was performed by decapitation. Immediate removal of both cochleae was performed. The cochleae were immediately stored in liquid nitrogen and then stored at -80°C. Analysis was performed within 4 weeks.

7. Radiation was delivered in a single dose using a Varian Clinac 2100C linear accelerator. The total dose of radiation was 70 Gy (7 krad), an amount consistent with a common, cumulative dose for treatment of head and neck cancer. A 6 megavolt X-ray radiation dose was delivered at 1000 rad/min at a distance of 61.6 cm from target to rat head (Figure 1). A 1.0 cm "bolus" tissue equivalent was placed over the rat head to achieve maximum dose depth to the cochleae. The Varian Clinac 2100C is a widely used, hospital-based linear

accelerator that is employed in the treatment of patients with head and neck cancer, as well as many other forms of neoplasm. Using standard light field collimation, the radiation field was restricted to a 3 cm by 2 cm window that was centered over both cochleae. Brainstem structures and eyes were excluded from the radiation field. All five animals in both groups survived.

8. Auditory brainstem response (ABR) testing was used to assess auditory threshold. Testing occurred immediately prior to radiation and again 24 hours later, immediately prior to sacrifice. All testing was performed with the animal in a double-walled IAC booth. Platinum/iridium needle electrodes were placed at the vertex (non-inverting) to a point directly below the ipsilateral pinna (inverting) with a ground electrode placed in the hind leg. ABR data collection was obtained with a Biologic Traveler system with an additional custom-made high frequency stimulator for 14000 Hz. ABR thresholds were measured in response to 100 μs clicks and tonebursts. Tonebursts were centered at frequencies of 1, 4, 8, and 14 kHz with a 2ms rise/fall for 1 kHz and a 1 ms rise/fall for 4, 8, and 14 kHz. All tonebursts had a 0 ms plateau gated by a Blackman envelope. Stimuli were presented at 10/s. An intensity series was obtained for each animal from 100 to 0 dB peak equivalent SPL (peSPL) for click stimuli and sound pressure level (SPL) for tonebursts in 10 dB decrements. The term peSPL means that the amplitude of the click stimulus from the pre-stimulus baseline to the first peak is equivalent to the SPL of a pure tone stimulus having the same pre-stimulus baseline-to-peak amplitude. For 14 kHz tonebursts, the intensity series was expanded down to –20 peSPL to accommodate the rats' lower threshold at that frequency. Threshold was defined as the lowest intensity capable of eliciting a replicable, visually detectable response. A total of 512 sweeps constituted each average. The recording epoch was 15 ms following stimulus onset. Responses were analogue filters with a 30-3000 Hz bandpass. Rectal temperature was monitored throughout recordings with animal temperature being maintained by a warming pad.

9. Each rat cochlea was stored in a separate, sealed 150 ml container marked with the specimen number and a designation of either "R" or "L" signifying the side from which the cochlea came. The cochleae were immediately placed in liquid nitrogen after removal from the cranium. After placement in container, the specimens were refrigerated at –80 °C. The specimens were removed from refrigeration only at the time of analysis of antioxidant enzyme activity. All cochlear tissue was homogenized using a manual Con Torque homogenizer. For analysis of superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase, the homogenized cochlear tissue was centrifuged and the supernatant was used as the tissue extract. For the malondialdehyde analysis, the homogenized cochlear tissue was used as the tissue extract.

10. Determination of superoxide dismutase (SOD, CuSOD, MnZnSOD) activity was measured at room temperature according to the method described in Misra H.P., Fridovich I. (1972) *J Biol Chem* 247:3170-3175. Tissue extract (100 microliters) was added to 880 µL (0.05 M, pH 10.2, 0.1 mM EDTA) sodium carbonate buffer. Twenty µL of 30 mM epinephrine (dissolved in 0.05% acetic acid) was added to the mixture and SOD was measured at 480 nm for 4 min on a Hitachi U2000 Spectrophotometer. The rate of the oxidation of epinephrine was calculated where linearity occurred. One unit of SOD activity was expressed as the amount of enzyme that inhibited the oxidation of epinephrine by 50 percent.

11. Determination of catalase (CAT) activity was determined at room temperature by a slight modification of the method described in Aebi, H. (1984) *Methods Enzymol* 105:125-126. Ten µL of ethanol was added per 100 µL of tissue extract (dissolved in 0.05 M, pH 7.0, 0.1 mM EDTA, phosphate buffer), and then placed in an ice bath for 30 minutes. Then, 10 µL of Triton X-100 RS was added. Ten µL of tissue extract was added to a cuvette containing 240 µL phosphate buffer and 250 µL (0.066 M) H_2O_2 (dissolved in sodium phosphate buffer) and measured spectrophotometrically at 240 nm for 30 seconds. A molar

extinction coefficient in $M^{-1}cm^{-1}$ was used to determine CAT activity. One unit of CAT activity was defined as the mmoles of H_2O_2 degraded/min/mg protein.

12. Determination of glutathione peroxidase (GSH-Px) activity was determined at 37°C by the method described in Flohe L., Gunzler W.A. (1984) *Methods Enzymol* 105:115-121. All reaction mixtures were dissolved in 0.05 M, pH 7.0, 0.1 mM EDTA phosphate buffer. A reaction mixture consisted of 550 µL phosphate buffer, 100 µL 0.01 M reduced glutathione (GSH), 100 µL 1.5 mM reduced nicotinamide adenine dinucleotide phosphate (NADPH), and 100 µL glutathione reductase (GR) (0.24 units). One hundred µL of the tissue extract was added to the reaction mixture and incubated at 37°C for 10 minutes. Then 50 µL of 12 mM t-butyl hydroperoxide was added to the tissue reaction mixture and measured spectrophotometrically at 340 nm for 180 seconds. A millimolar extinction coefficient in $mM^{-1}cm^{-1}$ was used to determine the activity of GSH-Px. One unit of activity was equal to the millimoles of NADPH oxidized/min/mg protein.

13. Glutathione reductase (GR) activity was determined at 37°C by the method described in Carlberg, I., Mannervik, B. (1985) *Methods Enzymol* 113:484-499. Fifty microliters of NADPH (2 mM) in 10 mM Tris-HCl buffer (pH 7.0) added in a cuvette containing 50 µL of oxidized glutathione (GSSG) (20 mM) in phosphate buffer (0.5 M, pH 7.0, 0.1 mM EDTA), and 800 µL of phosphate buffer were incubated at 37°C for 10 minutes. One-hundred µL of tissue extract was added to the NADPH-GSSG buffered solution and measured at 340 nm for 3 minutes. An extinction coefficient in $mM^{-1}cm^{-1}$ was used to determine the activity of GR. One unit of GR activity was equal to the millimoles of NADPH oxidized/min/mg protein.

14. The extent of lipid peroxidation was estimated by the concentration of thiobarbituric acid reactive products (*e.g.*, malondialdehyde (MDA)). Two volumes of tissue extract were homogenated and mixed with one volume of 30%

trichloroethane (TCA) and centrifuged at 5,000 g for 15 min. A portion of the supernatant and 0.5 ml of 1% thiobarbituric acid in 0.05 N NaOH was added to a loosely stoppered test tube and heated in boiling water for 10 min. The tubes were cooled under tap water and the absorption spectra recorded. Concentrations of thiobarbituric acid were determined using a molar extinction coefficient. Results were expressed in nmoles of MDA/mg protein.

15. Each animal's weight was measured in an Ohaus triple beam balance scale before administration of the anesthetic for the pre-test and again before the post-test 24 hours later.

16. ABR data were analyzed using a three factor analysis of variance (ANOVA) with one between subject factor (groups) and two within subject factors (frequency and pre- vs. post-test). Each dependent variable was analyzed independently. Tests subsequent to the ANOVA were carried out in accordance with the Tukey HSD procedure. The criterion for statistical significance for all measures was $p \leq 0.05$.

17. The degree of ABR threshold shift was not significantly different for the D-methionine and radiation group as compared to the radiation only group of rats. But, at 1kHz, 4 kHz, and 8 kHz, the D-methionine and radiation group tended to have a lower threshold shift as compared to the radiation only group of rats. No threshold shift was observed in the non-radiated control group.

18. The tympanic membrane and middle ear were fully exposed and examined within 5 minutes of sacrifice. At that time, no tympanic perforation, inflammation, necrosis, or effusion was noted in either group.

19. No mortality or morbidities, other than hearing loss, occurred in any group. No loss of appetite, coordination, significant weight loss, or diarrhea was observed.

20. The activity data in the cochlear tissue for the various enzymes are presented in attached Tables 1 to 5. These data demonstrate significant radiation-induced changes in the levels of activity of some antioxidant enzymes. For example, D-methionine administration before radiation exposure significantly increased the activity of glutathione peroxidase (GSH-Px), catalase (CAT), and superoxide dismutase (SOD) as compared to the control group and the radiation only group of rats. In addition, the malondialdehyde (MDA) levels were generally lower for the group treated with D-methionine as compared with the control and the radiation only group of rats.

21. Generally, these enzyme activities are important because they are factors involved in oxidative damage to cells from a variety of insults, including radiation. For example, active oxygen species (*e.g.*, superoxide, peroxide, hydroxyl radical) can be damaging and/or lethal to cells. SOD, CAT and GSH-Px convert these active oxygen species to water. SOD converts the superoxide radical into peroxide and CAT converts peroxide into water. GSH-Px is in the glutathione pathway and also converts peroxide into water by oxidizing the reduced form of glutathione (GSH) to glutathione disulfide (GSSG). When a balance between production of active oxygen species and their destruction is disturbed, the active oxygen species can oxidize other cell structures such as lipids. The malondialdehyde (MDA) levels indicate lipid peroxidation. Because the data presented in Tables 1, 3 and 4 show D-methionine administration significantly increases the activities of SOD, CAT and GSH-Px in the cochlear tissue, D-methionine aids the balance between production of active oxygen species and their destruction even when the animals are exposed to radiation. Further, these data suggest that cochlear free radical activity plays a role in radiation-induced hearing loss.

22. In sum, these findings suggest that radiation to the head and neck affect cochlear antioxidant mechanisms. The lack of obvious inflammatory changes in the middle ear at the time of dissection suggests that a process other

than inflammatory damage causes radiation-induced hearing loss. Likewise, the rigorous avoidance of brainstem irradiation afforded by this model makes it unlikely that the hearing loss was caused by damage to hearing centers in the brain. It seems possible, therefore, that the effects of radiation on cochlear free radical activity contribute significantly to the hearing loss that is incident to head and neck radiation treatment. The role of D-Methionine and other antioxidant co-factors then become particularly important as potential otoprotective agents.

24. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the Unites States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

Date: 11/17/05 _____

Table 1. GSH-Px concentration in cochlear tissues

Rat	Group 1 (control)	Group 2 (radiation)	Group 3 (D-met)
1	218.5	134.7	270.1
2	212.9	157.1	264
3	149	103.1	273.8
4	187.4	111.9	249.2
5	144.6	92.8	157.7
Mean	182.48	119.92	242.96
Std. Dev.	34.65122	25.90139	48.57616
Std. Err.	15.4965	11.58345	21.72392
% Control		65.71379	133.1434

Table 2. GR concentration in cochlear tissues

Rat	Group 1 (control)	Group 2 (radiation)	Group 3 (D-met)
1	67.8	44.9	76
2	63.6	82.1	66.3
3	58.2	34.2	32.5
4	49.8	65.4	67
5	55.6	49.8	34.7
Mean	59	55.28	55.3
Std. Dev.	6.989993	18.73411	20.19022
Std. Err.	3.12602	8.37815	9.029341
% Control		93.69492	93.72881

Table 3. CAT concentration in cochlear tissues

Rat	Group 1 (control)	Group 2 (radiation)	Group 3 (D-met)
1	109.7	39.8	44.2
2	76.4	37.6	116.7
3	77.2	31.8	38.3
4	76.8	23	95.1
5	40.7	50.4	67.6
Mean	76.16	36.52	72.38
Std. Dev.	24.41258	10.11988	33.38738
Std. Err.	10.91763	4.525749	14.93129
% Control		47.95168	95.03676

Table 4. SOD concentration in cochlear tissues

Rat	Group 1 (control)	Group 2 (radiation)	Group 3 (D-met)
1	35.2	26.4	78.3
2	67.4	31.2	69.8
3	39	32.3	93.7
4	40	72.5	80.2
5	77.4	31.9	43.3
Mean	51.8	38.86	73.06
Std. Dev.	19.21822	18.95423	18.71318
Std. Err.	8.594649	8.476591	8.368787
% Control		75.01931	141.0425

Table 5. MDA concentration in cochlear tissues

Rat	Group 1 (control)	Group 2 (radiation)	Group 3 (D-met)
1	1.27	0.939	1.1
2	1.06	1.62	1
3	0.93	1.93	0.565
4	1.44	1.52	1.12
5	1.03	0.818	1.08
Mean	1.146	1.3654	0.973
Std. Dev.	0.205743	0.471423	0.232583
Std. Err.	0.092011	0.210827	0.104014
% Control		119.1449	84.90401

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Examiner Rebecca Cook

DECLARATION OF PRASAD SUNKARA UNDER 37 CFR 1.132

I, Prasad Sunkara, hereby declare and state as follows:

1. I received a Ph.D in Biochemistry from the Indian Institute of Science, Bangalore, India. After completing a post doctoral fellowship in Cell Biology, I joined the faculty at M.D. Anderson Cancer Center in Houston, Texas, as an Assistant Professor of Cell Biology. I was employed at Marion Merrell Dow Research Institute in Cincinnati for 13 years and ended my time there as the Head of Tumor Biology wherein I was responsible for research and preclinical development of cancer and antiviral therapeutics. In all, I have more than 27 years of research and development management experience in cancer therapeutics at major pharmaceutical and biotech companies.

2. I am currently Chairman and Chief Executive Officer of Molecular Therapeutics, Inc.

3. Under my direction and control, pharmaceutical formulations containing D-methionine were evaluated as agents to reduce various side effects of radiation exposure.

4. In one study for prevention of radiation induced oral mucositis in mice, female C3H mice weighing approximately 24 grams were exposed to five doses of 6Gy radiation for a total dose of 30 Gy. There were four groups of mice used in the study (1) the non-radiated control group; (2) the radiated control

group; (3) a pre-treatment group receiving 150 mg/kg of MRx-1024 (an oral suspension of D-methionine) by oral gavage 1 hour before each dose of radiation; and (4) a post-treatment group receiving 150 mg/kg of MRx-1024 by oral gavage 1 hour after each dose of radiation. Rats representing the results of this study are shown in attached Figure 1. Hair loss at the site of radiation in the radiated control group was observed. But, hair loss was not observed for rats in the pre-treatment or post-treatment groups.

5. In addition to the above study, another study evaluating the survival of jejunal crypt cells per jejunal circumference was undertaken. In this study, groups of 5 mice were exposed to one dose / day for 5 consecutive days resulting in a total whole body irradiation varying from 5 Gy to 20 Gy. For each whole body radiation dose of 5 Gy, 10 Gy, 15 Gy, and 20 Gy, there were two groups of mice. One group of mice received 300 mg/kg of an oral suspension of D-methionine by oral gavage 1 hour before the radiation dose. Another group of mice did not receive D-methionine and received radiation alone. To assay crypt stem cell survival, mice were killed 66 hours after the last radiation dose. A 2 cm section of jejunum was fixed in neutral buffered formalin and prepared for histological examination. The number of regenerating crypts in the jejunal cross-section was microscopically counted at 100X magnification on sections stained with hematoxylin and eosin (H&E) and cut at a thickness of 4 μm. To construct radiation survival curves, the number of regenerating crypts was converted to the number of surviving cells per jejunal circumference by applying a Poisson correction for crypts regenerating from more than 1 stem cell. This method is described in more detail in Mason et al., Int. J. Radiation Oncology Biol. Phys., Vol. 44, No. 5, pp. 1125-1135, 1999. A graph of the total whole body radiation dose (in Gy) versus the surviving jejunal crypt cells per jejunal circumference resulting from this study is shown in attached Figure 2. This graph shows statistically significant increased survival of jejunal crypt cells in the mice treated with D-methionine for total whole body radiation doses of 10 Gy and 15 Gy, and generally increased survival of jejunal crypt cells in the mice treated with D-

methionine for all total whole body radiation doses. The increased survival of jejunal crypt cells per jejunal circumference in the mice treated with D-methionine indicates that D-methionine decreases the gastrointestinal effects of whole body radiation.

6. I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the Unites States Code, and that such willful false statements may jeopardize the validity of the above-referenced application or any patent issuing thereon.

Date: 11-15-05